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CLONING OF RAT AND MOUSE P_{2Y} PURINOCEPTORS

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The cellular responses to ATP are mediated by specific high-affinity receptors designated as P_2 purinoceptors, five subclasses of which have been defined pharmacologically - P_{2X}, P_{2Y}, P_{2U}, P_{2T}, and P_{2Z}. A cDNA clone encoding a rat P_{2Y} purinoceptor was isolated from an insulinoma cDNA library. The 373-amino acid rat P_{2Y} purinoceptor sequence has 85.7% and 37.8% identity with chicken P_{2Y} and rat P_{2U} purinoceptors, respectively. The sequence of the mouse P_{2Y} purinoceptor was also determined and there was 97.1% amino acid identity with the corresponding rat sequence. RNA blotting studies showed that rat P_{2Y} purinoceptor mRNA was expressed at variable levels in many tissues including heart, brain, spleen, lung, liver, skeletal muscle and kidney, although it was not detected in testis. The cloned rat P_{2Y} purinoceptor was expressed in *Xenopus laevis* oocytes and possessed the properties expected for this receptor subtype.

Extracellular ATP elicits diverse biological responses in every organ and tissue system that has been studied (1,2). It affects cardiovascular and immune function, acts as a fast excitatory transmitter in the nervous system, and stimulates platelet aggregation, contractile responses in smooth muscle cells, secretion of Cl ions by respiratory epithelial cells and hepatic glycogenolysis. ATP is also a potent secretagogue in endocrine and neuroendocrine cells including insulin-secreting ß-cells. The cellular responses to ATP are mediated by P_2 purinoceptors, five subclasses of which have been defined pharmacologically (P_{2X}, P_{2Y}, P_{2U}, P_{2T}, and P_{2Z}). Of these, the P_{2Y} and P_{2U} purinoceptors signal through G proteins and P_{2X} receptors are ligand-gated ion channels. Mouse, rat and human P_{2U} purinoceptors, and chicken and turkey P_{2Y} receptors have been cloned and characterized, and shown to be members of the superfamily of G protein-coupled receptors with seven transmembrane segments (3-7). Two distinct but structurally-related rat P_{2X} receptors have also been cloned, and these are members of a new class of ligand-gated ion channels (8,9).

Extracellular ATP stimulates insulin release both *in vivo* and *in vitro* and studies using the perfused pancreas system, isolated islets, and insulinoma cells suggest that islets and ß-cells have

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both P_{2Y} and P_{2X} purinoceptors (10-15). In addition to responding to ATP, ß-cells also secrete ATP which is an important constituent of insulin secretory granules (16) suggesting that ATP has an autocrine or paracrine role in regulating insulin secretion. As a first step in elucidating the role of extracellular ATP in regulating insulin secretion and other physiological functions, we have cloned and characterized rat and mouse P_{2Y} purinoceptors.

MATERIALS AND METHODS

Cloning of Rat and Mouse P_{2Y} Purinoceptors

Standard molecular biology methods were carried out as described (17). First-strand cDNA was prepared with 1 µg of RNA and 20 pmol of oligo $d(T)_{16}$ primer (Perkin Elmer, Norwalk, CT) in 20 µl of a solution containing (in mM) 50 Tris-Cl (pH 8.4), 75 KCl, 3 $MgCl_2$, 10 dithiothreitol, 0.125 dNTP, 2 units of RNasin (Promega, Madison, WI), and 200 units Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Purinoceptor-related sequences in RINm5F insulinoma cell RNA were amplified using the primers

5'-AGCATCCT(C/G)TTCCTCAC(C/G)TG-3' and

5'-GAG(G/T/C)A(T/C)(C/G)GGGTC(G/A)A(C/G)(A/G)CA(G/A)CTGTT-3'

selected from homologous regions of transmembrane domains 3 and 7 of the chicken P_{2Y} and mouse and human P_{2U} purinoceptors (the sequence of rat P_{2U} purinoceptor was reported subsequently). The PCR included: 40 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. PCR products of approximately 600 bp were selected by electrophoresis in a 1% low melting point agarose gel, cloned into the *Hinc*II site of pGEM-3Z (Promega) and sequenced (Sequenase™ version 2.0, United States Biochemical, Cleveland, OH). Rat P_{2Y} purinoceptor cDNA clones were isolated from a RINm5F cDNA library by hybridization with ^{32}P-labeled insert from prP2Y-2. The sequence of mouse P_{2Y} purinoceptor was determined from the sequences of overlapping PCR fragments obtained by amplification of MIN6 cDNA with the primer pairs:

1. 5'-CCTCTCGTCGCGGTCTGTCCTT-3' and CACTGACATAAATGGCATTC-3'
2. 5'-GAATGCCATTTATGTCAGTG-3' and 5'-TGTCAACATAACTGTCATTAT-3'
3. 5'-TGTATGTGCTCACCCTACCAG-3' and 5'-TCCTGCCTTCACAAACTTGTG-3'.

At least two cloned PCR products were sequenced and additional clones were sequenced if the amino acid sequences of mouse and rat P_{2Y} purinoceptors differed.

RNA Blotting

A rat multiple tissue Northern blot (Clontech, Palo Alto, CA) was hybridized with ^{32}P-labeled insert from prP2Y-2.The blot was exposed to X-ray film in the presence of an intensifying screen at -80 °C for 2 days.

Expression of Cloned Rat P_{2Y} Purinoceptor in *Xenopus laevis* Oocytes

A 2.1-kb *Eco*RI fragment (corresponding to nucleotides 1-2108) of λRY1 was subcloned into the *Eco*RI site of pGEM-3Z to generate pRY1. pRY1 DNA was linearized with *Sal*I and synthetic capped rat P_{2Y} purinoceptor mRNA (cRNA) was prepared using a kit and T7 RNA polymerase (Riboprobe Gemini Systems, Promega). Stage IV/V oocytes were prepared as described (18). The follicular cell layer was removed by enzymatic treatment with collagenase and manual peeling and oocytes were incubated for 24 hr at 16°C in OR-2 media. Oocytes were injected with 50 nl (50 ng) of cRNA and incubated for 2-4 days at 16°C in OR-2 media.

Current Measurements in *Xenopus* Oocytes

Whole oocyte currents were recorded using the conventional two-microelectrode voltage-clamp technique. The bath solution contained (in mM) 90 NaCl, 2 KCl, 2 $CaCl_2$, 5 HEPES, pH 7.2. Nucleotides and other reagents were dissolved directly in the bath solution just before the experiments. The pH was adjusted to 7.2 using NaOH and HCl after addition of other reagents. Electrodes contained 3 M KCl and had resistances from 0.2 to 1 MΩ. All experiments were conducted at room temperature. After electrode penetration, oocytes were allowed to stabilize for at least 10 min. The membrane currents were conditioned by a two-step voltage-clamp protocol stepped to -100 mV (1 s) followed by +50 mV (4 s) from the holding potential of -40 mV which was repeated every 20 s. Current and voltage signals were recorded using a voltage-clamp amplifier (CA-1, Dagan Instruments, Minneapolis, MN). Results were analyzed with pClamp software (Axon Instruments).

Fura-2 Loading of *Xenopus* Oocytes and Fluorescence Measurements

Oocytes expressing rat P_{2Y} purinoceptors were injected with 50 nl of a solution containing (in mM) 19 NaCl, 52 KCl, 10 HEPES, pH 7.2, and 0.2 fura-2AM (Molecular Probes, Eugene, OR) and then incubated at 16°C for 30-60 min before study. The oocyte was illuminated alternately at 340 and 380 nm using a 75-W xenon arc lamp and fluorescence emission was collected with a Hamamatsu C2400 video camera (Hamamatsu Photonics, Hamamatsu City, Japan).

Drugs

ATP, ADP, UTP, 2-methylthio-ATP, α,ß-methylene-ATP, 2-chloro-ATP and reactive blue 2 were obtained from RBI (Natick, MA).

RESULTS

Cloning of Rat and Mouse P_{2Y} Purinoceptors

cDNAs encoding metabotropic ATP receptors expressed in RINm5F insulinoma cells were amplified using PCR and degenerate oligonucleotide primers. The primer sequences were selected from conserved regions present in transmembrane segments 3 and 7 of the chicken P_{2Y} (4) and mouse (3) and human P_{2U} purinoceptors (5). PCR amplification of RINm5F insulinoma cDNA generated a discrete product of about 600 bp which was cloned and sequenced. Of the six clones sequenced, four encoded a protein having 86% amino acid identity with chicken P_{2Y} purinoceptor suggesting that they were the rat homolog of this receptor. The sequences of the other two clones, which were identical, did not have any features of G protein-coupled receptors or correspond to any known protein in the GenBank/EMBL database.

The insert from the putative rat P_{2Y} purinoceptor cDNA clone, prP2Y-2, was used to screen a RINm5F cDNA library by hybridization. One clone, λRY1, having an insert of 3.6 kb was isolated and sequenced. The longest open reading frame in λRY1 encoded a protein of 373 amino acids (Fig. 1). This open reading frame was preceded by an in-frame stop codon beginning 18 nucleotides upstream of the ATG. The 619 bp 5'-untranslated region of the rat P2Y purinoceptor cDNA sequence contained five other ATG's, each closely following by a stop codon.

There was 85.7% amino acid identity and 90.9% similarity between rat and chicken P_{2Y} purinoceptors, and 37.8% identity and 53.3% similarity between the rat P_{2Y} and P_{2U} purinoceptors (Fig. 1).

The sequence of mouse P_{2Y} purinoceptor was determined by sequencing a series of overlapping PCR products generated by amplification of MIN6 insulinoma cell (19) cDNA with primers based on the rat cDNA sequence. There was 97.1% amino acid identity between the rat and mouse sequences (Fig. 1). All sequence differences between the rat and mouse proteins were confirmed by sequencing additional PCR products obtained from a second PCR reaction.

Tissue Distribution of Rat P_{2Y} Purinoceptor mRNA

RNA blotting showed a single transcript of 4.2 kb that was present in heart, brain, spleen, lung, liver, skeletal muscle and kidney (Fig. 2). Rat P_{2Y} purinoceptor mRNA could not be detected in testis. The P_{2Y} purinoceptor mRNA was highly expressed in heart and skeletal muscle and at lower levels in the other tissues examined. The pattern of expression of P_{2Y} purinoceptor mRNA in adult rat tissues was different from that noted in chickens where this mRNA was detected by RNA blotting in brain and skeletal muscle but not in heart, spleen, lung, spleen or kidney (4).

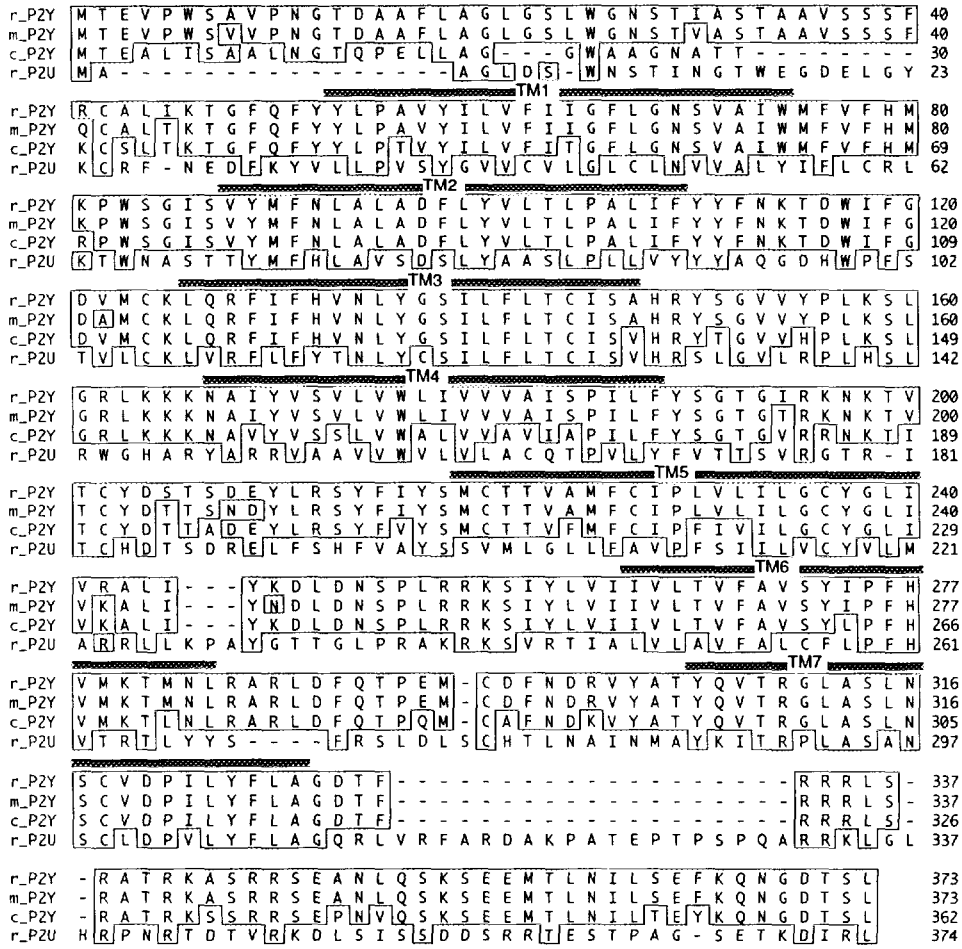
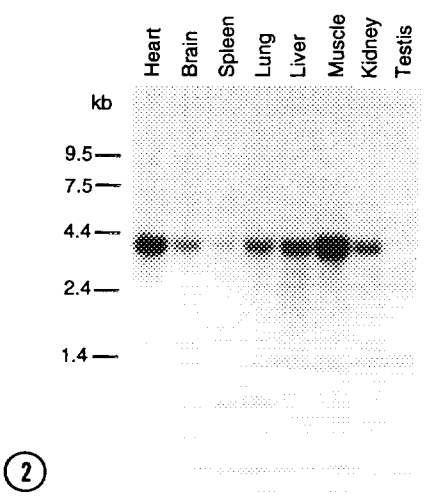


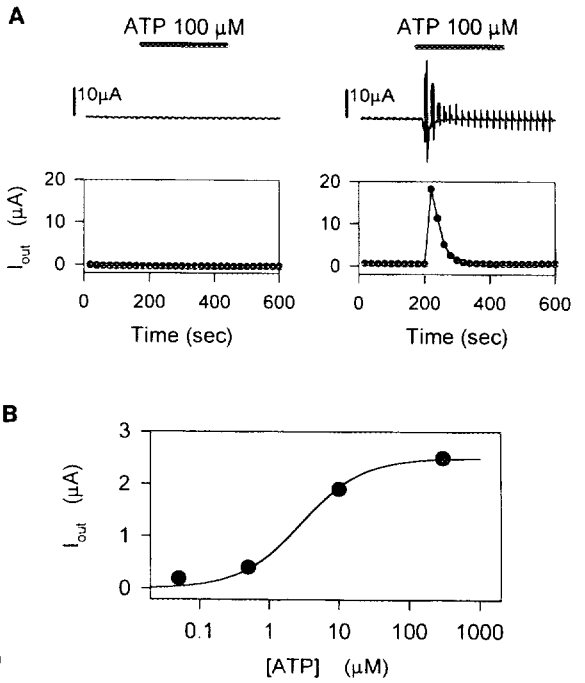
Fig. 1. Sequences of rat and mouse P_{2Y} purinoceptors and comparison with chicken P_{2Y} and rat P_{2U} purinoceptors. Amino acid residues that match the rat sequence are boxed. The rat and mouse P_{2Y} purinoceptor sequences are noted by r_P2Y and m_P2Y, the chicken P_{2Y} sequence is c_P2Y, and rat P_{2U} is r_P2U. The seven putative α-helical transmembrane regions are noted as TM1 to TM7. Gaps introduced to generate this alignment are noted by *dashes*. The sequences of the rat and mouse P_{2Y} purinoceptors have been deposited in the GenBank/EMBL database with accession numbers U22830 and U22829, respectively.

Characterization of the Cloned Rat P_{2Y} Purinoceptor

The properties of the cloned rat P_{2Y} purinoceptor were studied following expression in *Xenopus* oocytes. Activation of P_{2Y} purinoceptors *in vivo* leads to an increase in $[Ca^{2+}]_i$ due to inositol-1,4,5-trisphosphate mediated release of intracellular Ca^{2+} stores (1,2,20). Application of extracellular ATP evoked a membrane current in oocytes injected with rat P_{2Y} purinoceptor cRNA that was not observed in uninjected oocytes (Fig. 3). The current was rapidly activated upon the application of 100 μM ATP, quickly reached a peak value, and then decayed within 2-3 min. Recovery of the current following ATP application required at least 10 min washout. This ATP-



(2)



(3)

Fig. 2. Tissue distribution of rat P_{2Y} purinoceptor mRNA. A RNA blot containing 2 μg of poly(A)+ RNA from various rat tissues was hybridized with the insert from prPY-2 (nucleotides 1031-1604 of the cDNA sequence). RNA size markers are shown on the left.

Fig. 3. Expression of cloned rat P_{2Y} purinoceptor in *Xenopus* oocytes. A. The upper panels show current recordings of a control uninjected oocyte (left) and an oocyte injected with 50 ng of rat P_{2Y} purinoceptor cRNA (right). The lower panels show the average measured outward current during the +50 mV voltage step with the corresponding recording time. The bars above the current records indicate the periods during which 100 μM ATP was applied to the bath solution. B. The relationship between the maximal outward current as a function of external ATP concentration in a single oocyte expressing the rat P_{2Y} purinoceptor.

activated current was observed in 11 of 32 injected oocytes using four different cRNA preparations. The effects of other purinoceptor ligands on membrane current were also studied (1,2,21). This current could be activated by 10 μM 2-chloro-ATP and 2-methylthio-ATP which are P_{2Y} purinoceptor-selective agonists (Fig. 4) and was inhibited by the P_{2Y} purinoceptor antagonist reactive blue 2 (Fig. 5). Addition of the P_{2U} receptor-selective agonist UTP (10 μM) had no effect although current was observed on application of 300 μM UTP (data not shown). There was also no response to the P_{2X} purinoceptor specific agonist 10 μM α-ß-methylene-ATP. These results are consistent with the cloned receptor being a P_{2Y} purinoceptor.

The effect of extracellular ATP on $[Ca^{2+}]_i$ was also measured directly by imaging fura-2 fluorescence. In 2 of 2 oocytes identified by voltage clamp to have a robust response to extracellular ATP, application of 100 μM ATP caused a rapid increase in fluorescence at 340 nm and a corresponding decrease at 380 nm (Fig. 5). ATP had no effect on control oocytes (data not shown).

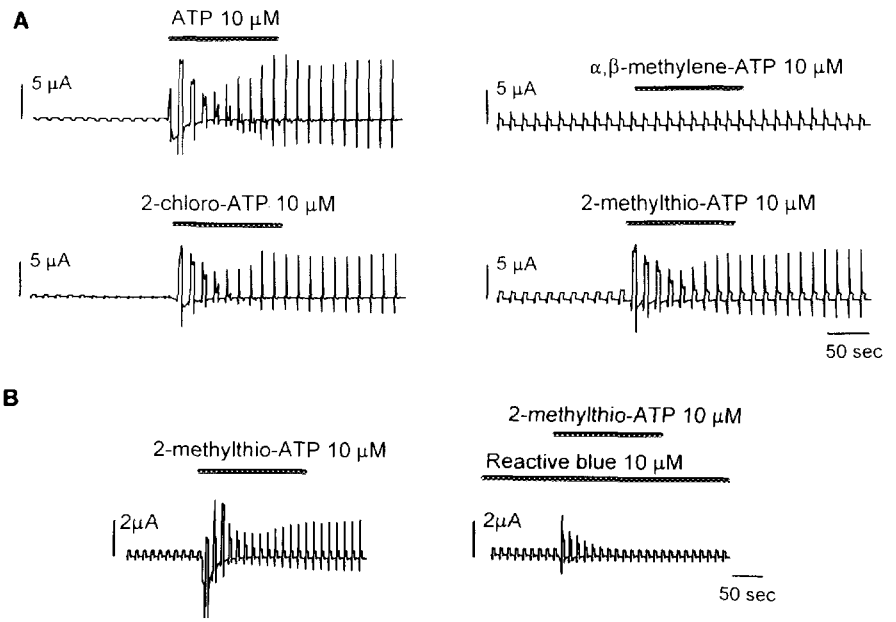


Fig. 4. Selectivity of cloned rat P_{2Y} purinoceptor. A. Current responses of a single oocyte injected with 50 ng of rat P_{2Y} purinoceptor cRNA to the application of 10 μM ATP, 2-chloro-ATP, α,ß-methylene-ATP, and 2-methylthio-ATP are shown. The time intervals between each recording were 50, 40 and 20 min, respectively. B. Effect of reactive blue on current recordings of an oocyte injected with 50 ng of rat P_{2Y} purinoceptor cRNA in response to application of 10 μM 2-methylthio-ATP.

DISCUSSION

Extracellular ATP affects many physiological responses including insulin secretion (1,2, 10-15). The presence of high concentrations of ATP and ADP in insulin secretory granules that are co-secreted with insulin suggests a role for these nucleotides in the autocrine or paracrine regulation of pancreatic islet function (16). In this regard, pancreatic islets are composed of a heterogeneous population of ß-cells with differential sensitivity to glucose (22). ATP may amplify the effects of glucose on adjacent pancreatic ß-cells and thereby integrate the overall insulin secretory response of the islet.

Insulinoma cells express both metabotropic and ionotropic purinoceptors (12-14) implying that individual ß-cells themselves express more than one type of ATP receptor. Pharmacological studies indicate that P_{2Y} purinoceptors may mediate at least some of the effects of extracellular ATP on insulin secretion *in vivo* (15). The contribution of ligand-gated ATP receptors such as P_{2X} purinoceptors to the islet response to glucose is less clear. The cloning of P_{2Y} and P_{2U} purinoceptors and two forms of P_{2X} purinoceptors from mammalian cells will allow the role of each of these receptors in mediating the effects of ATP on cell function to be examined directly.

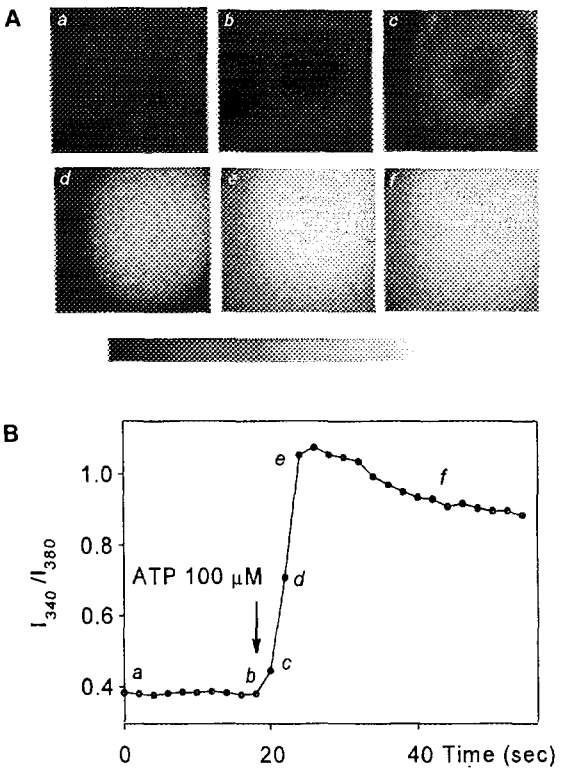


Fig. 5. Cloned rat P_{2Y} purinoceptor mediates release of $[Ca^{2+}]_i$. A. Images of fura-2 fluorescence (ratio 340/380) of a single oocyte injected with 50 ng of rat P_{2Y} purinoceptor cRNA. B. Average 340/380 ratio at different times. The signal of the whole area of the oocyte was averaged. The arrow indicates the time at which 100 μM ATP was applied. Background fluorescence was subtracted prior to calculation of 340/380 ratio. *a-f* correspond to the fura-2 fluorescence at the intervals noted in A.

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